The Plant Cell, Vol. 20: 88–100, January 2008, www.plantcell.org © 2008 American Society of Plant Biologists

Requirement of B2-Type *Cyclin-Dependent Kinases* for Meristem Integrity in *Arabidopsis thaliana* [W][OA]

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To maintain proper meristem function, cell division and differentiation must be coordinately regulated in distinct subdomains of the meristem. Although a number of regulators necessary for the correct organization of the shoot apical meristem (SAM) have been identified, it is still largely unknown how their function is integrated with the cell cycle machinery to translate domain identity into correct cellular behavior. We show here that the cyclin-dependent kinases *CDKB2;1* and *CDKB2;2* are required both for normal cell cycle progression and for meristem organization. Consistently, the *CDKB2* genes are highly expressed in the SAM in a cell cycle–dependent fashion, and disruption of *CDKB2* function leads to severe meristematic defects. In addition, strong alterations in hormone signaling both at the level of active hormones and with respect to transcriptional and physiological outputs were observed in plants with disturbed *CDKB2* activity.

INTRODUCTION

All above-ground parts of a plant, such as stem, leaves, flowers, and fruits, are derived from a small number of pluripotent stem cells residing in the shoot apical meristem (SAM). Therefore, plants are absolutely dependent on the presence of these cells to complete their lifecycle. This strict dependence is reflected in the evolution of a robust regulatory system that is able to integrate local meristematic cues with global signals, which relay environmental parameters and the overall growth status of the organism. Forward genetic screens have uncovered some of the key regulators for SAM organization, and their localized expression has helped to identify important functional domains within this tissue. These studies revealed that due to the activity of the homeodomain transcription factor WUSCHEL (WUS), the core of the meristem acts as an organizing center, which is essential for induction of stem cell fate in the overlying cells (Laux et al., 1996; Mayer et al., 1998). The stem cells in turn express *CLAVATA3* (*CLV3*), which codes for a short secreted peptide molecule and acts to restrict *WUS* expression in deeper layers (Fletcher et al., 1999; Ito et al., 2006; Kondo et al., 2006). Therefore, in *clv3* mutants, *WUS* expression is expanded and stem cells proliferate inappropriately, while in plants with compromised *WUS* activity, stem cell fate and, thus, meristem function is terminated prematurely. Cells within the central stem cell domain divide slowly, but once they are displaced into the peripheral zone, they divide more rapidly before being incorporated into newly forming organs at the flanks of the meristem. This function is dependent on the activity of another homeodomain transcription factor, SHOOT MERISTEMLESS (STM), which is expressed throughout the meristem and acts to inhibit cell differentiation (Long et al., 1996). The complex pattern of cellular behavior within the meristem highlights the need for careful balancing of cell division and differentiation in the various domains to preserve the structure and function of this tissue. Thus, signals from meristem regulators must be perceived and accurately interpreted by the cell cycle machinery.

The eukaryotic cell cycle is a highly regulated process that relies on a series of discrete checkpoints to ensure proper DNA replication and successful cytokinesis. Progression through the individual steps of the cycle is dependent on the activation of cyclin-dependent kinases (CDKs) by interaction with their specific cyclin partners. While a single CDK is sufficient to execute the cell cycle in yeast, higher eukaryotic organisms, such as plants and animals, have expanded complements of cell cycle regulators. The *Arabidopsis thaliana* genome contains 38 *Cyclin*-related genes, many of which are expressed in a cell cycle–specific manner (Menges et al., 2005). Among the 29 *CDK*-related sequences, *CDKA;1* is the archaetypical *CDK* in *Arabidopsis*, as demonstrated by its ability to complement the yeast *cdc28* mutant (Ferreira et al., 1991). Like most other *CDK* genes, it is expressed at a similar level throughout the cell cycle, while the plant-specific B-type *CDKs* are unique in having a strictly cell cycle–dependent transcription profile (Segers et al., 1996; Magyar et al., 1997; Menges and Murray, 2002; Menges et al., 2005). The roles of individual plant cell cycle regulators have been assayed genetically using gain- and loss-of-function strategies. Overexpression of a dominant-negative *CDKA;1* allele resulted in plants with fewer but dramatically larger cells (Hemerly et al., 1995),

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www.plantcell.org/cgi/doi/10.1105/tpc.107.054676

whereas complete loss of *CDKA;1* function caused gametophytic lethality, highlighting its essential nature (Iwakawa et al., 2006; Nowack et al., 2006). Plants expressing a dominant-negative allele of *CDKB1;1* displayed normal overall morphology but also had fewer and larger cells, which had higher ploidy levels than wild-type controls. In addition, these plants had specific defects in stomatal cell division (Boudolf et al., 2004a, 2004b). Originally identified based on their ability to complement yeast mutants, the D-type cyclins are among the most extensively studied CDK interaction partners in plants (Soni et al., 1995). Significant acceleration of plant growth by *CYCLIN D* overexpression was accompanied by only subtle changes in SAM structure (Cockcroft et al., 2000; Boucheron et al., 2005); likewise, additional cell divisions could be induced in the SAM without compromising its organization (Dewitte et al., 2003).

These studies have suggested that plant architecture is remarkably resistant to the manipulation of many cell cycle regulators, and it has been proposed that cell divisions are merely subordinate in the execution of developmental programs (Kaplan and Hagemann, 1991; Gutierrez, 2005). However, since meristematic cells proliferate at different rates within the various subdomains, it would appear that the cell cycle machinery is able to integrate organizing signals to maintain a functional stem cell niche. To address this paradox, we have searched for core cell cycle regulators that are active in the meristem and whose expression is dependent on the functional organization of this tissue.

RESULTS

B2-Type *CDKs* Are Expressed in Functional SAMs

To identify regulators required for both meristem function and cell cycle control, we surveyed *Arabidopsis* microarray data sets for cell cycle–related genes that show preferential expression in the shoot apex and that are sensitive to disruption of key meristematic regulators. In addition, we scored for cell cycle–dependent mRNA accumulation. Using these criteria, we found a pair of duplicated B-type *CDKs*, *CDKB2;1* and *CDKB2;2*, that are regarded as core cell cycle regulators and fulfilled our mRNA expression criteria. In the AtGenExpress developmental data set (Schmid et al., 2005), expression of these genes was highest in samples corresponding to the shoot apex (Figure 1A) but was strongly reduced in shoot apices of 3-d-old *wus* and *stm* mutant seedlings, which fail to maintain a proper SAM (Figure 1B). By contrast, *CDKB2;1* and *CDKB2;2* mRNA levels were slightly elevated in *clv3* mutants, which have an enlarged meristem (Figure 1B). Furthermore, both genes showed a peak in expression during the G2-to-M phase transition of the cell cycle (Menges et al., 2003) (Figure 1C). Additional information about the presumed phylogenetic relationships and expression patterns of all *Arabidopsis CDKs* can be found in Supplemental Figure 1 online.

To resolve the localization of *CDKB2* mRNAs at a cellular level, we performed in situ hybridizations on 12-d-old seedlings and confirmed strong cell cycle–dependent expression of both transcripts in cells of the SAM and young leaves (Figure 3A). A weaker cell cycle–dependent expression was seen in root tips (Figure 3F; see Supplemental Figure 2A online). Since *CDKB2;1* and *CDKB2;2* cDNAs share 86% percent identity on the nucleotide

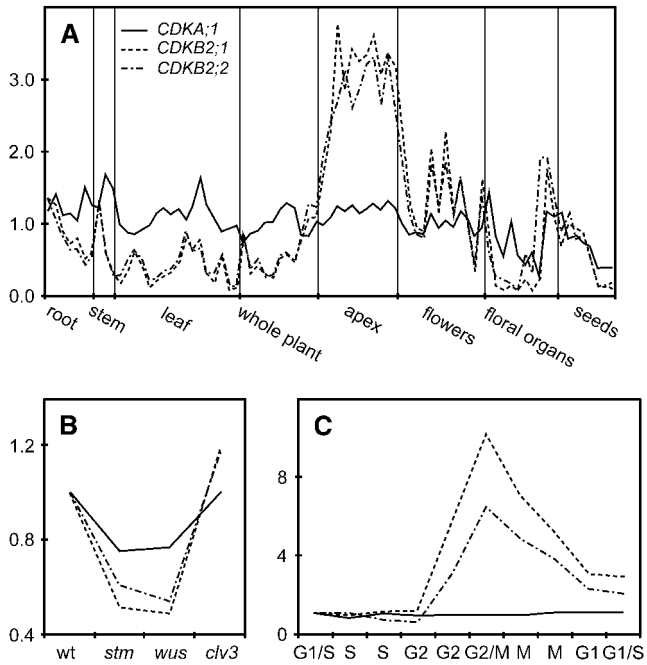

Figure 1. Relative Expression Levels of *CDKA;1*, *CDKB2;1*, and *CDKB2;2*.

(A) The *CDKB2s* were highly expressed in the apex, whereas *CDKA;1* was expressed at similar levels across all tissues. Expression levels were normalized to the per-gene average across all samples.

(B) *CDKB2* expression was reduced in *stm* and *wus* mutants, which have impaired meristem function, while their transcript levels were increased in *clv3* mutants with enlarged meristems. Expression levels were normalized to the wild-type control.

(C) The *CDKB2s* showed a peak in expression level at the G2/M transition, while *CDKA;1* expression did not change in response to the cell cycle phase. Transcript abundance was normalized to the expression level at the time of cell cycle block release.

Microarray data were extracted from the AtGenExpress compendium (Menges et al., 2003; Schmid et al., 2005) and represent biological triplicates in **(A)** or biological duplicates in **(B)** and **(C)**.

level, RNA probes corresponding to the full-length coding sequences likely detect both transcripts simultaneously. To distinguish between the two *CDKs*, we prepared probes from the untranslated regions of both genes, which are unrelated in sequence. Despite weaker signals, which likely result from short probe lengths, we confirmed that both *CDKB2* transcripts are expressed in cells of the SAM (see Supplemental Figure 3 online).

Requirement of *CDKB2* Function for Proper Organization of the SAM

To test whether the identified *CDKB2s* play a role in the SAM, we analyzed plants in which expression levels of *CDKB2;1* and/or *CDKB2;2* were modified. As appropriate T-DNA insertion lines were not available for both genes, we used artificial microRNAs (amiRNAs) to assess loss-of-function phenotypes (Schwab et al., 2006; details on amiRNA design can be found in Supplemental

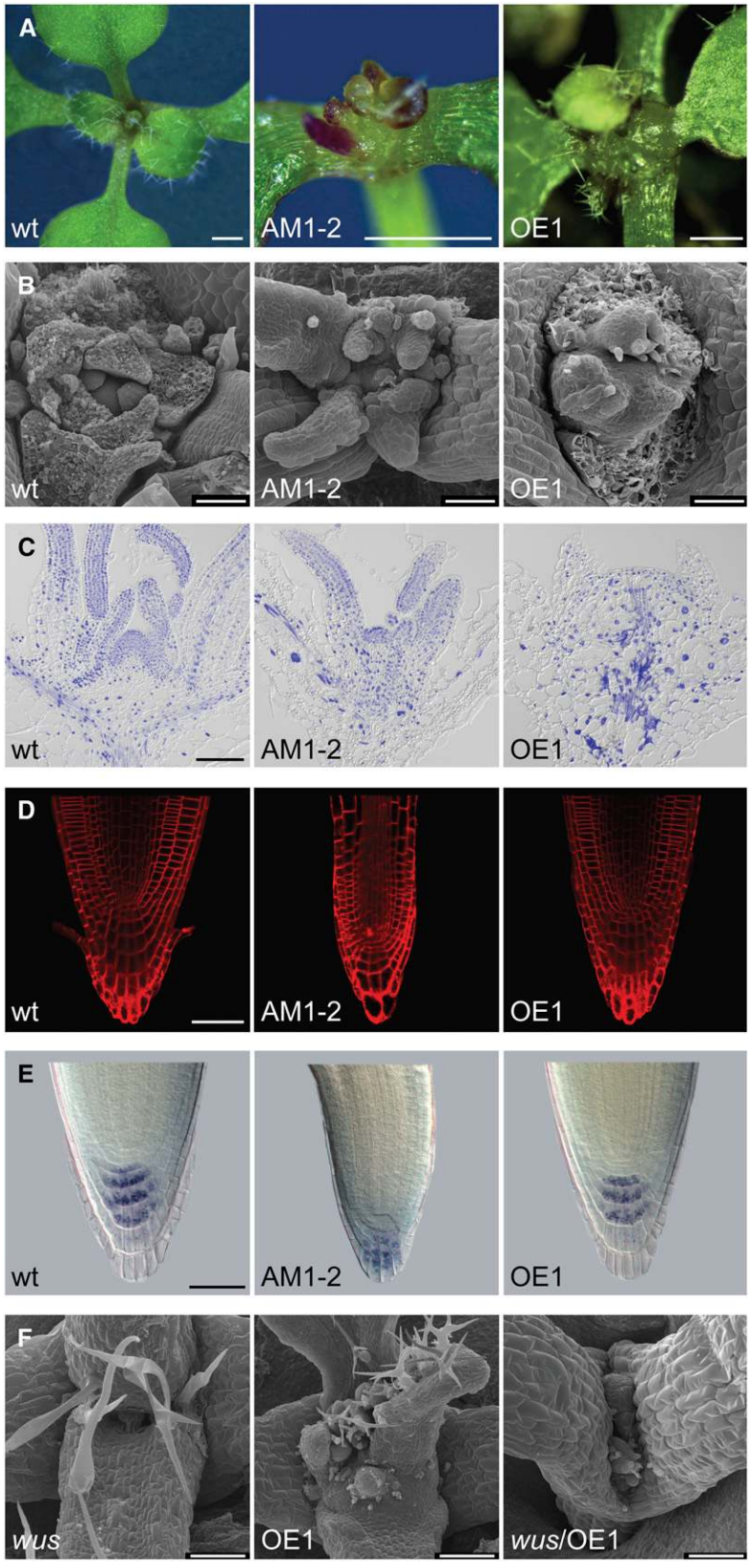

Figure 2. Phenotypes of AM1-2 Double Knockdown and *35S:CDKB2;1* (OE1) Plants.

Figure 4 online). Plants harboring constructs in which the cauliflower mosaic virus *35S* (*35S*) promoter drove expression of amiRNAs targeting *CDKB2;1* (AM1) or *CDKB2;2* (AM2) individually did not display aberrant phenotypes. However, when we introduced both constructs simultaneously, we recovered double transformants showing dwarfism, abnormal structure of the shoot meristem, and phyllotaxis defects. To confirm that silencing of both *CDKB2* genes caused the observed phenotypes, we designed a third amiRNA that targeted both transcripts for degradation (AM1-2). Thirty-five percent of the resulting T1 transgenic plants recapitulated the phenotype seen in AM1/AM2 double transformants and showed severely disturbed meristem structure and strong overall growth inhibition (Figures 2A to 2C).

We observed a similar, albeit weaker, phenotype in plants overexpressing either *CDKB2;1* or *CDKB2;2* cDNAs from the *35S* promoter. While meristematic activity was immediately blocked after germination in AM1-2 double knockdown lines, plants carrying *CDKB2* overexpression constructs were able to initiate two to four true leaves before the meristem arrested. After a few days of apparent growth arrest, AM1-2 plants started to initiate radialized organs at multiple positions of the meristem, whereas in *35S:CDKB2;1* (OE1) plants, cells of the meristem expanded improperly and formed a bulge before organs emerged at irregularly spaced foci (Figures 2A to 2C). A summary of phenotype frequencies is given in Supplemental Table 1 online.

In contrast with AM1-2 lines, in which development irreversibly terminated, mildly affected *35S:CDKB2;1* plants were able to make the transition to flowering and produced seeds, which allowed us to establish a stable single insertion transgenic line. The offspring from these plants segregated for mild and severe phenotypes dependent on the copy number of the transgene. Hemizygous plants showed defects in phyllotaxis and organ spacing, while homozygous plants recapitulated the phenotypes of strong T1 lines described above (Figures 2A to 2C; see Supplemental Figure 5 online).

To determine whether the defects in meristematic organization are restricted to the SAM, we investigated the structure of the root apical meristem (RAM) in overexpression and double knockdown lines. Roots of *35S:CDKB2;1* plants were indistinguishable from wild-type roots in terms of root length and RAM organization, while the number of lateral roots was reduced (Figures 2D and 2E; see Supplemental Figure 6 online). Meristem organization was also largely unaffected in roots of AM1-2 plants, despite the fact that they consisted of fewer cells. By comparison with control lines, we found that kanamycin selection contributed little to the smaller root size of AM1-2 plants, indicating that it, like the shoot phenotype, was caused by the transgene. Due to the early manifestation of the shoot phenotype in AM1-2 plants, we were unable to distinguish whether the root phenotype was caused directly by aberrant *CDKB2* activity in the root or whether it was an indirect effect of the reduced shoot size. Thus, we cannot rule out that *CDKB2* disruption has a negative impact on root growth. However, since the structure of the root meristem was maintained in both *CDKB2* overexpressors and double knockdown plants, it appears that the RAM was much more resistant to perturbations in *CDKB2* activity than the SAM.

To investigate the basis for the similar phenotypes in overexpression and double knockdown plants, we analyzed *CDKB2* expression by quantitative real-time RT-PCR (q-RT-PCR) and in situ hybridization. As expected, mRNA abundance of both *CDKB2* transcripts in the shoot and root was reduced in AM1-2 double knockdown plants (Figures 3A and 3F; see Supplemental Figure 7 online). In *35S:CDKB2;1* plants, overall *CDKB2;1* mRNA levels were increased in both root and shoot, although endogenous *CDKB2;1* and *CDKB2;2* expression was reduced (see Supplemental Figure 7 online). In situ hybridization revealed that the strong cell cycle–dependent expression of *CDKB2;1* in discrete cells of the meristem was lost in these plants and was replaced by a uniform but weaker expression in all cells (Figures 3A and 3F). These findings suggest that strong, cell cycle–dependent expression of *CDKB2;1* is required for its full function and that the similar phenotypes of knockdown and overexpression lines are likely caused by a disruption of this pattern.

CDKB2s Play a Role in Both Cell Cycle Regulation and Meristem Organization

Our analysis of tissue sections not only allowed us to asses the changes in expression levels of *CDKB2;1* but also revealed abnormal cellular organization and morphology within the apices of both double knockdown and overexpression lines. While the

Figure 2. (continued).

The genotype is indicated at the lower left of each picture.

(A) Apices of 15-d-old plants. At this age, the emergence of multiple rosettes from the disorganized apex was observed in both AM1-2 and OE1 plants. Bars = 500 μm.

(B) Scanning electron micrographs of apices from 12-d-old seedlings. The strict organization seen at the wild-type apex was disrupted in both AM1-2 and OE1 plants. Bars = 90 μm.

(C) Overlays of differential interference contrast bright-field images and 4′,6-diamidino-2-phenylindole nuclear stainings. Nuclei appear blue. The dome-shaped meristem-like structures found in AM1-2 and OE1 plants contained fewer cells than wild-type meristems. In addition, several nuclei of AM1-2 and OE1 plants were abnormally expanded, and in OE1 plants, these were accompanied by abnormally large cells. Bar = 100 μm.

(D) FM4-64 staining of root tips. The organization of the root meristem is maintained in both AM1-2 and OE1 plants. Bar = 50 μm.

(E) Lugol staining of root tips. Starch grains are deposited normally in the columella cells in both transgenic lines. Bar = 50 μm.

(F) Phenotype of *wus*/*35S:CDKB2;1* (OE1) double mutant plants. Homozygous *wus* mutant plants produced the first set of fully developed true leaves before terminating in a flat apex with no discernible meristem. Homozygous OE1 plants produced the first set of true leaves, followed by swelling of the apex and initiation of multiple irregularly spaced rosettes. *wus*/OE1 double homozygous plants displayed developmental arrest and completely failed to produce organs. Bars = 200 μm.

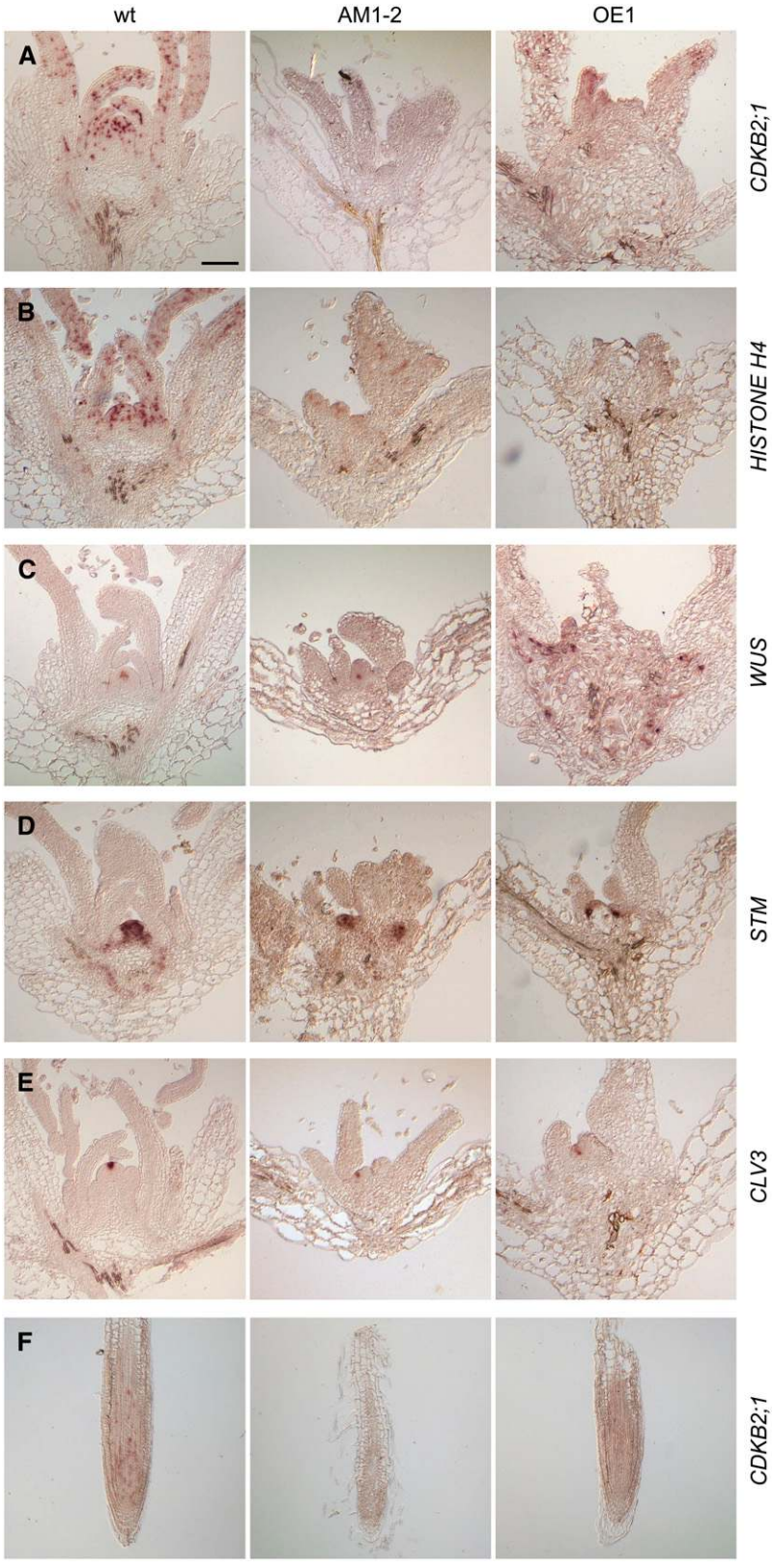

Figure 3. In Situ Hybridizations.

basic dome shape of the meristem was maintained in our transgenic lines, the strict organization into three distinct tissue layers was disrupted (Figure 2C). In addition, cell number was significantly reduced in the meristems of knockdown and overexpression lines, and cells were dramatically enlarged in apices of *35S:CDKB2* plants. Using 4′,6-diamidino-2-phenylindole staining, we also observed abnormally large nuclei in double knockdown and overexpression lines (Figure 2C).

To asses whether the cell cycle was affected in these apices, we analyzed *HISTONE H4* expression as a marker for cells in S-phase and found that while *HISTONE H4* mRNA could be detected in a pattern similar to *CDKB2* transcripts in the wild type, expression was strongly reduced in overexpression and double knockdown plants (Figure 3B). We then measured nuclear DNA content as an independent marker for cell cycle activity, since nonproliferating plant cells that undergo differentiation expand their DNA content by endoreduplication. To control for potential differences in leaf age and size, wild-type seedlings with and without the oldest set of leaves were included. Nuclei from both overexpression and double knockdown lines had significantly higher nuclear DNA content than either wild-type sample (Figure 4), consistent with a misregulation of the G2–M transition. The reduced cell cycle activity within the meristem and the increase in nuclear DNA content demonstrated that disruption of *CDKB2* function causes defects in cell division control.

Since both knockdown and overexpression lines had multiple centers of organ initiation, we asked whether meristem-organizing genes are ectopically activated in these functional niches. In situ hybridization on overexpression and knockdown plants showed that expression of *WUS*, *STM*, and *CLV3* colocalized with multiple spots of apparent organ initiation within a single meristem (Figures 3C to 3E; see Supplemental Figure 8 online). This observation suggests that there might be feedback from the cell cycle to the network of meristem organizers and that ectopic activation of meristem regulators could be required for the formation of functional centers of activity. To test this hypothesis, we crossed the *CDKB2;1* overexpression line to *wus* mutants and observed segregation of three distinct phenotypes in the F2 generation. The first phenotypic class corresponded to *wus* mutants (Laux et al., 1996), in which meristematic activity ceases after the formation of the first true leaves, resulting in a flat apex without a discernible SAM (Figure 2F). The second phenotypic class displayed all features of severely affected *CDKB2;1* overexpression plants, which produce one set of leaves followed by disorganization of the meristem and reinitiation of organ formation from multiple sites (Figure 2F). Intriguingly, the third class exhibited a new phenotype. In these plants, the SAM never acquired activity and development was terminated after expansion of the cotelydons (Figure 2F). Genotyping showed that these plants were *wus* mutants and contained two copies of the *35S:CDKB2;1* transgene, while *wus* mutants hemizygous for the transgene were similar to nontransgenic mutants (see Supplemental Figure 9 online). Consistently, 12 (expected 14) out of 56 *wus* mutants displayed the completely arrested phenotype. Taken together, these results demonstrate that ectopic activation of *WUS* is required for formation of functional meristematic centers in plants with compromised *CDKB2* activity and conversely that *CDKB2s* are required for the remaining organ forming capacity of *wus* mutants.

CDKB2 Activity Is Required for Proper Plant Hormone Signaling

To elucidate the molecular basis for the phenotypes observed in lines with disrupted *CDKB2* function, we performed expression-profiling experiments using Affymetrix Ath1 microarrays. Double knockdown and overexpression seedlings were compared with wild-type controls 10 d after germination in two biological replicates consisting of pools of six to 15 individuals each. The overall molecular signature of the two lines was similar, consistent with our findings at the phenotypic and cellular levels, where both lines showed meristems with fewer cells, increased ploidy levels, and multiple centers of meristematic activity. Accordingly, statistical testing using the Rank products algorithm (Breitling et al., 2004) identified 219 genes whose expression changed significantly in both of the *CDKB2* transgenic lines (Figure 5A; see Supplemental Data Set 1 online). The direction of change for all these genes was the same in double knockdown and overexpression lines (Figure 5A), confirming that *35S:CDKB2;1* plants can be regarded as mild *CDKB2* loss-of-function lines.

Analyzing the functional categories of the differentially expressed genes, we found many transcripts of the plant hormone signaling and response class among the top ranks. *THIONIN 2.1* (AT1G72260) stood out as the most highly induced gene, changing 50- and 350-fold in knockdown and overexpression plants, respectively (Figure 5B). *THIONIN 2.1* has been identified as a jasmonate (JA)–induced transcript (Bohlmann et al., 1998), and several transcripts coding for enzymes involved in JA synthesis

Figure 3. (continued).

All images were taken at the same magnification. Bar = 100 μm.

(A) In the wild type, *CDKB2;1* was expressed at the apex in a spotty pattern characteristic of a cell cycle–regulated gene. Expression could not be detected in AM1-2 plants, whereas OE1 plants showed a weaker expression in a larger number of cells.

(B) *HISTONE H4* was expressed in many cells of wild-type apices, but its expression was attenuated in both AM1-2 and OE1 plants.

(C) to **(E)** A single center of meristematic activity with *WUS*, *STM*, and *CLV3* expression was seen in wild-type apices. By contrast, AM1-2 and OE1 plants contained multiple foci of *WUS*, *STM*, and *CLV3* expression, consistent with the emergence of multiple rosettes observed. See Supplemental Figure 8 online for images of serial sections.

(F) In roots, *CDKB2;1* was expressed in a spotty pattern in cells near the root tip. This is consistent with the root digital in situ data available (Brady et al., 2007; see Supplemental Figure 2 online). Like in the shoot, expression could not be detected in AM1-2 plants, whereas OE1 plants showed a weaker expression in a larger number of cells.

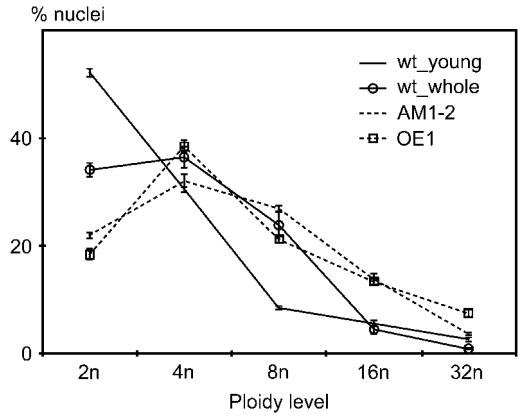

Figure 4. Nuclear DNA Content of AM1-2 Double Knockdown and *35S:CDKB2;1* (OE1) Plants.

Ploidy of AM1-2 and OE1 plants was compared with wild-type control seedlings with (wt_whole) and without (wt_young) the oldest set of leaves. The horizontal axis indicates the genome copy number, and the vertical axis shows the percentage of nuclei counted. Error bars represent SE. In both AM1-2 and OE1 plants, the relative number of 2n nuclei was reduced, while the number of 16n nuclei was increased compared with either wild-type sample, indicating a shift toward higher genome copy numbers in plants with altered *CDKB2* activity.

were also elevated in both *CDKB2* lines (Figure 5B). JA plays important roles in stress and pathogen signaling as well as in the regulation of growth and development (Farmer and Ryan, 1992; Sanders et al., 2000; Stintzi and Browse, 2000; Turner et al., 2002). It has also been shown that JA signaling is modulated by the cell wall status (Ellis et al., 2002), suggesting the changes in cell morphology observed in the *CDKB2* disruption lines might trigger JA responses.

At the other end of the spectrum, the primary cytokinin response genes were most prominent among the genes expressed at lower than wild-type levels, and three A-type *ARRs* (*ARR6*, *ARR15*, and *ARR16*) were found among the 32 most reduced transcripts (Figure 5B; see Supplemental Data Set 1 online). Cytokinin is essential for meristem function, since it is required for cell cycle progression at multiple checkpoints (Zhang et al., 1996; Riou-Khamlichi et al., 1999), and removal of active cytokinin from the plant by overexpression of a cyctokinin oxidase leads to meristem termination (Werner et al., 2003). A-type *ARRs* have been implicated in a negative feedback regulation of cytokinin response (Kiba et al., 2003; To et al., 2004), and we have recently shown that *ARR7* and *ARR15* play prominent roles in meristem regulation (Leibfried et al., 2005).

To directly test whether hormone-dependent developmental programs are affected in our *CDKB2* lines, we performed tissue regeneration assays. Wild-type root explants can be induced to form callus, root, or shoot in vitro, depending on the relative levels of auxin and cytokinin in the regeneration medium. This response is dependent on the proper function of hormone signaling pathways and on developmental regulators (Reinert and Bajaj, 1977; Endrizzi et al., 1996; Kakimoto, 1996). While roots of *CDKB2;1* overexpression plants were largely normal at the time of explantation, they were not able to respond properly to the hormone stimuli leading to regeneration and differentiation in wild-type roots. On callus induction medium, all wild-type root explants responded with formation of calli at the wounding sites. By contrast, explants from overexpression plants did not form calli but produced irregular outgrowths from young lateral roots (Figures 6A and 6B). The induction of roots by auxin was completely inhibited in these lines (Figures 6C and 6D). Explants growing on shoot induction medium with 0.5 to 5 μM cytokinin (2-iP) responded by limited cell proliferation and weak tissue greening, whereas wild-type explants formed clearly discernible green foci at 5 μM (Figures 6E to 6J). Increasing the cytokinin concentration in the shoot induction medium further to 50 μM 2-iP caused wild-type explants to produce callus, whereas

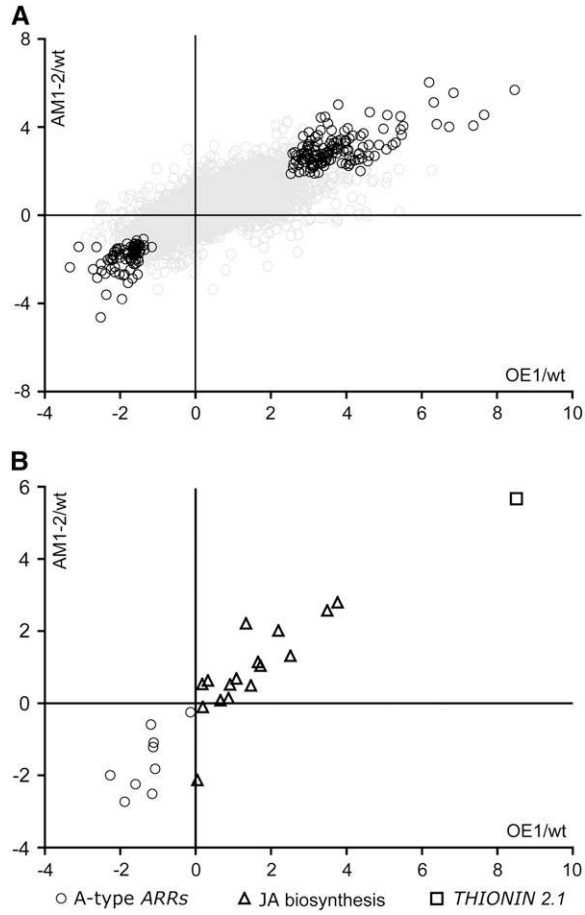

Figure 5. Molecular Phenotypes of AM1-2 Double Knockdown and *35S:CDKB2;1* (OE1) Plants by Global Expression Analysis.

The horizontal axis shows the log2-transformed OE1/wild type expression ratio, and the vertical axis indicates the log2-transformed AM1-2/wild type expression ratio.

(A) Expression ratios of all genes (gray circles). The 219 genes that changed significantly in both conditions (percentage of false positives < 10%) are highlighted in black.

(B) Expression ratios of A-type *ARRs*, JA biosynthesis genes (JA biosynthesis), and *THIONIN 2.1*.

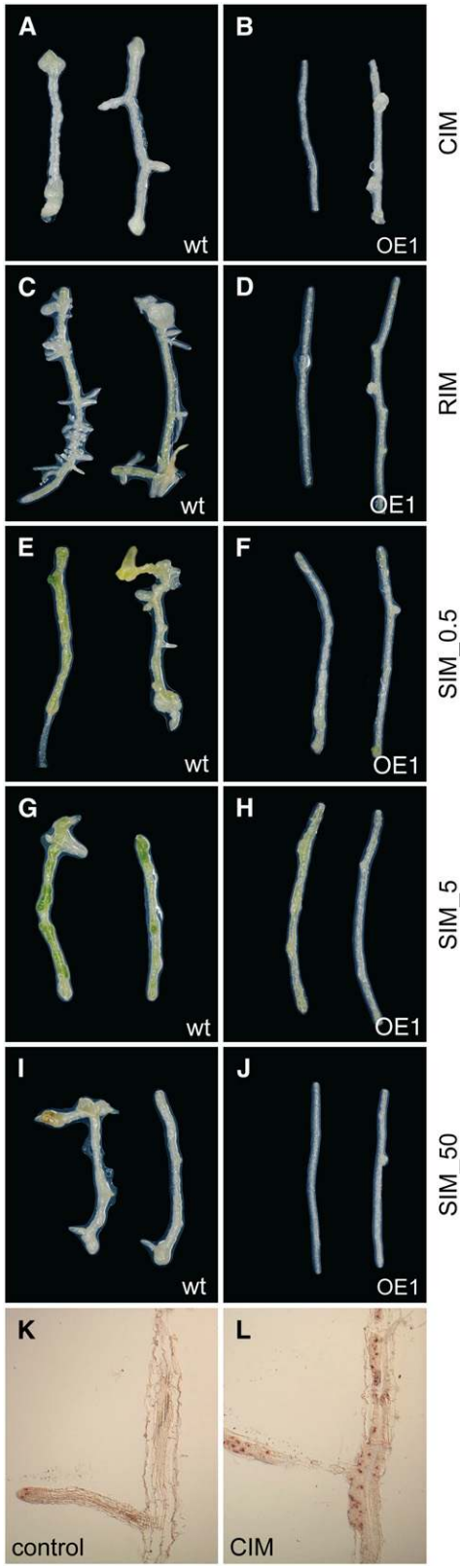

Figure 6. Hormone Response of Wild-Type and *35S:CDKB2;1* (OE1) Tissue.

(A) to **(J)** Wild-type and OE1 root explants were subjected to hormone treatments to assess their regeneration capacity. Plant genotype and growth medium are indicated. CIM, callus induction medium; RIM, root induction medium; SIM_0.5, shoot induction medium with 0.5 μM 2-iP; SIM_5, shoot induction medium with 5 μM 2-iP; SIM_50, shoot induction medium with 50 μM 2-iP. OE1 roots were recalcitrant to tissue regeneration and, in contrast with wild-type explants, did not form calli, roots, or green foci in response to hormone treatment.

(K) and **(L)** Wild-type root explants after 5 d of incubation on either hormone-free (control) or CIM plates. Whereas *CDKB2* expression in the control explants could only be detected in root tips, *CDKB2* expression was detected throughout proliferating callus tissue in the explants incubated on CIM.

overexpression explants showed no response (Figures 6I and 6J). We detected *CDKB2* transcripts in proliferating calli by in situ hybridization, consistent with a role for these genes in tissue regeneration (Figures 6K and 6L). Bearing in mind that *CDKB2* activity is not necessary for cell divisions in the root in general, these results demonstrate that normal *CDKB2* activity is essential for the initiation of coordinated developmental programs in response to hormonal stimuli and suggest that the phenotypes observed in plants with disrupted *CDKB2* function could at least in part be caused by the inability of cells to properly interpret hormone signals.

To distinguish whether the defects in hormone signaling we observed on the functional and transcriptional levels are due to changes in hormone levels or if they are brought about by a direct transcriptional modulation of response genes, we quantified hormone content in our *CDKB2* disruption lines. Consistent with the former explanation, we found that auxin levels were significantly elevated in overexpression and knockdown lines (Figure 7). By contrast, the bioactive cytokinins, *trans*-zeatin (tZ), tZ-riboside, and tZ-5′monophosphate, were reduced, whereas the levels of inactive zeatin-*O*-glucosides were increased in overexpression seedlings (Figure 7). These results are consistent with the observed changes in gene expression and demonstrate that *CDKB2* disruption plants suffer from a severe perturbation of the level of bioactive hormones.

DISCUSSION

In the course of evolution, the machinery controlling the eukaryotic cell cycle has undergone substantial changes. While unicellular organisms, such as yeast, rely on the activity of a singe *CDK*, higher land plants contain a plethora of *CDKs*. Among those, the B-type *CDKs* appear to be plant specific, and their origin can be traced back to the unicellular green algae *Ostreococcus tauri*. This member of a lineage that is ancestral to vascular plants has a single B-type *CDK*, which is regulated in a cell cycle–dependent manner similar to that of *Arabidopsis CDKB1s* and is able to complement the yeast *cdc28* mutant (Corellou et al., 2005; Robbens et al., 2005). This function has been assumed by *CDKA;1* in *Arabidopsis*, but B-type *CDKs* are still integral parts of the *Arabidopsis* cell cycle machinery as evidenced by the sharing of interaction partners, such as CYCD4;1, CDK inhibitors, and CKS1 by A- and B-type CDKs (Ferreira et al., 1991; De Veylder

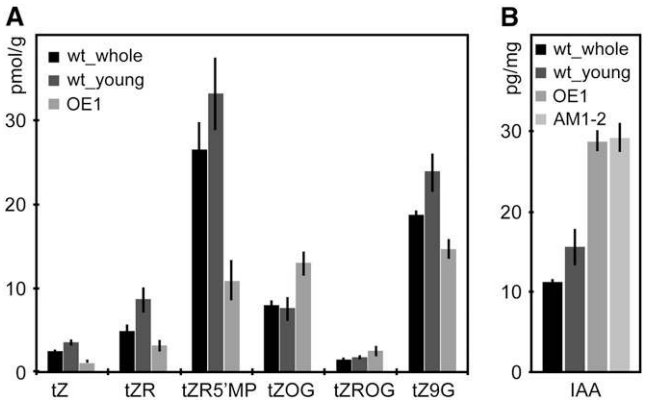

Figure 7. Hormone Content of *35S:CDKB2;1* (OE1) and AM1-2 Double Knockdown Plants.

Hormone levels of AM1-2 and OE1 plants were compared with wild-type control seedlings with (wt_whole) and without (wt_young) the oldest set of leaves.

(A) Quantification of *trans*-zeatin (tZ), its precursors tZ riboside (tZR) and tZ riboside 5′ monophospate (tZR5′MP), and conjugates tZ *O*-glucoside (tZOG), tZ riboside *O*-glucoside (tZROG), and tZ 9-glucoside (tZ9G). The bioactive nonglycosylated forms tZ, tZR, and tZR5′MP were present at lower levels in OE1 plants than in the wild-type controls, whereas the inactive *O*-glycosylated forms tZOG and tZROG were increased.

(B) Quantification of indole-3-acetic-acid (IAA). Both AM1-2 and OE1 plants contained significantly more IAA than either wild-type control. Error bars indicate SD. Cytokinin quantifications were performed on biological quadruplicates, and biological triplicates were used for IAA measurements.

et al., 1997; Boudolf et al., 2001; Kono et al., 2003; Nakai et al., 2006). The duplication of B-type *CDKs* could have allowed them to take on more specific roles, such as those related to multicellularity. Consistent with such a hypothesis, *CDKB1;1* is highly expressed in specialized cells that form stomatal complexes and is required for their development (Boudolf et al., 2004a), while we have now shown that *CDKB2s* are active in the SAM and necessary for its function.

Coordinated cell divisions are a prerequisite for the formation of organized multicellular tissues, but how much cell divisions actively contribute to the shaping of an organism is still a matter of debate (Gutierrez, 2005; Ramirez-Parra et al., 2005; Fleming, 2006; Inzé and De Veylder, 2006). The cellular theory claims that cell divisions are the main driving force of development, whereas the organismal theory proposes that cells are merely slaves to a higher-level developmental plan (Kaplan and Hagemann, 1991). To elucidate which of the principles underlie plant growth and development, cell division rates have been altered through manipulation of cell cycle regulator activity. Unfortunately, lethality has precluded the use of knockout alleles for many of the core regulators, such as *CDKA;1* and *RBR* (Ebel et al., 2004; Iwakawa et al., 2006; Nowack et al., 2006). However, overexpression of functional and/or dominant-negative alleles of core cell cycle regulators has allowed cell division rates to be modified in planta. The effects of these manipulations on overall plant morphology reported so far have been mild, supporting the organismal theory (Hemerly et al., 1995; Doerner et al., 1996; Cockcroft et al., 2000; Boudolf et al., 2004b). For example, manipulation of *CDKB1s* and *CDKA;1*, the regulators most closely related to the *CDKB2s*, by overexpression of dominant-negative alleles did not lead to disturbed meristem structure (Hemerly et al., 1995; Boudolf et al., 2004b). By contrast, we now show that interfering with *CDKB2* function not only causes abnormalities in cell cycle progression, but also in meristem organization. These phenotypes were not only observed in plants in which endogenous expression is knocked down by amiRNAs, a method new to the study of cell cycle regulators, but also in overexpression lines. This suggests that the defects are not merely a consequence of our knockdown strategy. While we certainly cannot rule out that knocking down other cell cycle regulators by amiRNAs would produce phenotypes similar to those seen for the *CDKB2s*, the results from interfering with the related *CDKB1s* and *CDKA;1* by dominant-negative strategies suggest that the function in meristem organization is not shared by all cell cycle regulators.

The organizational activity of the *CDKB2s* is tightly integrated with the function of classical meristem regulators. Consistently, a strong additive effect was seen upon introducing the *wus* mutant allele into plants with reduced *CDKB2* activity. These plants maintain some meristematic activity despite the perturbations in cell cycle control; however, when remaining organizational cues were removed by a mutation in *WUS*, the meristem was permanently arrested. This result demonstrates that meristem organization and cell division rates need to be coordinated for proper meristem function. Thus, our results are in line with the view that cell cycle regulators may serve as targets for the integration of cell proliferation with differentiation, morphogenesis, and growth (Gutierrez, 2005) and highlight the *CDKB2s* as such potential targets within the shoot meristem. The observed resistance of the root meristem to *CDKB2* disruption could be explained by the activity of redundant regulators in the root, which are absent from the shoot. Alternatively, the regulatory machinery of the root meristem could rely on factors different from the *CDKB2s* to integrate meristem organization and cell cycle control, in line with the differences observed in meristem development between root and shoot. Variations in root and shoot responses have also been observed in overexpression of the CDK inhibitor KRP2, where leaves and lateral roots were severely affected, whereas main root growth was uninhibited (De Veylder et al., 2001; Himanen et al., 2002).

Further support for a tight integration of cell cycle control and developmental programs comes from the finding that plant hormone signaling is strongly modulated at multiple levels in plants with disrupted *CDKB2* function. Auxin and cytokinin levels are known to control the cell cycle and many developmental programs throughout the plant (Skoog and Miller, 1957; Riou-Khamlichi et al., 1999; Himanen et al., 2002), and the levels of both hormones were changed in response to loss-of-*CDKB2* function. A striking example for the action of auxin and cytokinin is tissue regeneration, where the auxin/cytokinin ratio not only determines cell division rates, but also the developmental fate of the tissue (Skoog and Miller, 1957). We have found that tissue with disrupted *CDKB2* function cannot translate these stimuli into a proper developmental output, despite the fact that it was normal at the time of explantation. Thus, the developmental

block observed is not merely a consequence of an inability of cells to proliferate, but rather underlines the importance for a coordination of cell cycle and developmental programs by hormonal signals. In the SAM, the central role of plant hormones in these processes has been highlighted by the identification of the *pasticcino* and *tumorous shoot development* mutants, which have disorganized meristems and altered cytokinin sensitivity (Frank et al., 2002; Harrar et al., 2003). In addition, cytokinin biosynthesis in the meristem is stimulated by *STM*, whereas *WUS* is thought to modulate cyctokinin response by directly repressing a subset of the A-type *ARR* primary cytokinin response genes (Leibfried et al., 2005; Yanai et al., 2005). Both the hormonal requirements and the meristem organizing factors differ between shoot and root meristems, and this could explain why disruption of *CDKB2* activity had such a severe effect on the SAM, while RAM structure remained intact.

Our results demonstrate that cell cycle control by *CDKB2s* is essential for proper shoot meristem development and that hormones such as auxin and cytokinin might play an important role in relaying information between nodes of the regulatory network. Whether the *CDKB2s* act at the level of signal interpretation or in translating developmental programs into cellular behavior and whether there are other cell cycle regulators with similar functions represent intriguing questions for future study.

METHODS

Cloning of *CDKB2* amiRNA and Overexpression Constructs

To create the *35S:CDKB2;1* and *35S:CDKB2;2* constructs, *CDKB2;1* and *CDKB2;2* cDNAs were PCR amplified with primers adding Gateway B1 and B2 sites to their open reading frames. The resulting PCR products were recombined into the pDONR221 vector and subsequently into a vector containing the *35S* promoter in front of a Gateway cassette. The amiRNAs were created as previously described (Schwab et al., 2006) using Gateway tailed primers. All primer sequences are listed in Supplemental Table 2 online.

Plant Material

All plants were in the Columbia (Col-0) background and were grown in continuous light at 23°C and 65% relative humidity.

RNA Extraction and Expression Quantification

Double knockdown and overexpression seedlings showing the phenotypes described were compared with the wild type 10 d after germination in two biological replicates consisting of pools of 6 to 15 individuals each. For the double knockdown plants, pools of T1 plants grown on soil and selected with Basta were used, whereas pools of plants from a segregating T2 population grown on soil without selection were used for the overexpression line. RNA extraction and microarray analyses using the Affymetrix ATH1 platform were performed as described (Schmid et al., 2003). The data have been deposited at ArrayExpress (EMBL-EBI) under accession number E-MEXP-1100. Expression estimates were derived by GC-RMA at standard settings implemented in R. We determined significant changes on a per-gene level by applying the Rank products algorithm (Breitling et al., 2004) using 1000 permutations and a false discovery rate cutoff of 10%. For q-RT-PCR, total RNA was extracted using RNeasy Mini columns (Qiagen), and reverse transcription was performed with 1 μg of total RNA using the RevertAid first-strand cDNA synthesis kit (Fermentas). q-RT-PCR amplification was performed in the presence of the double-stranded DNA binding dye SYBR Green (Molecular Probes) and monitored in real time with the Opticon continuous fluorescence detection system (MJ Research). Amplification of *TUBULIN BETA-2* served as cDNA loading control. Primer sequences are listed in Supplemental Table 3 online.

Genotyping

The *wus* mutants were genotyped using a derived cleaved amplified polymorphic sequence marker. Oligos 5′-GTAGTAAAGTTCTTTGAGGATTTTGGTTT-3′ and 5′-AGTCGAATCAAACACACATG-3′ were used for amplification, and the PCR product was then digested with *Mja*IV (Hpy8I). The *35S:CDKB2;1* transgene copy number was measured by q-RT-PCR. To amplify the internal genomic control, oligos 5′-GCTATCCACAGGTTAGATAAAGGAG-3′ and 5′-GAGAAAGATTGTGTGAGAATGAAA-3′ were used. The *35S:CDKB2;1* transgene was quantified using oligos 5′-CACGAGGAGCATCGTGGAAA-3′ and 5′-GTGAGGATCACGAGCGAGCA-3′.

In Situ Hybridizations and Microscopy

Scanning electron microscopy was performed as previously described (Schmid et al., 2003). In situ hybridization was done in accordance with standard protocols (Weigel and Glazebrook, 2002). FM4-64 (Molecular Probes) staining of roots was done by mounting roots directly in FM4-64 working solution (4 μg/mL) and viewing the stained samples with a confocal microscope. Starch granules in the columella root cap were visualized with 1% Lugol solution. Seedlings were stained for 1 min, rinsed with water, cleared with chloral hydrate, and photographed.

Ploidy Measurements

Nuclei from whole seedlings or from seedlings without the oldest set of true leaves were extracted and labeled using the CyStain PI Absolute P (05-5022; Partec) kit according to the manufacturer's instructions. Stained samples (biological triplicates) were analyzed with the BD LSRII flow cytometer, and the data were acquired with BD FACS DiVA software (BDBiosciences). More than 3800 nuclei were counted per sample.

Hormone Measurements

Quantification of IAA

Seedlings were pooled in triplicates, weighed, and frozen in liquid nitrogen for quantification of free IAA content. The frozen sample containing between 13 and 54 mg of tissue (fresh weight) was homogenized in 0.5 mL of 50 mM sodium phosphate buffer, pH 7.0, containing 0.02% diethyldithiocarbamic acid (antioxidant) and 500 pg 13C6-IAA internal standard, using the Retsch vibration mill (Retsch) and a 3-mm tungsten carbide bead at a frequency of 30 Hz for 2 min. The sample was then incubated for 15 min at +4°C with continuous shaking. The pH was adjusted to 2.7 with 1 M HCl, and the sample was purified by solid phase extraction on a 500-mg Isolute C8-EC column (International Sorbent Technology) conditioned with 2 mL of methanol and 2 mL of 1% acetic acid. The column was washed with 2 mL of 10% methanol in 1% acetic acid and eluted with 2 mL 70% methanol in 1% acetic acid, and the sample was evaporated to dryness. The sample was dissolved in 0.2 mL of 2-propanol and 1 mL of dichloromethane, and IAA was methylated by adding 5 μL 2 M trimethylsilyl-diazomethane in hexane (Aldrich) and incubating the sample at room temperature for 30 min. Five microliters of 2 M acetic acid in hexane was added to destroy excess diazomethane, and the sample was then evaporated to dryness. The methylated sample was then trimethyl-silylated and analyzed by gas chromatography–selected reaction monitoring–mass spectrometry as described (Edlund et al., 1995).

Quantification of Cytokinin

The procedure used for cytokinin analysis was a modification of a previously described method (Novak et al., 2003). Frozen plant material (~0.1 g fresh weight) was extracted in Bieleski buffer using a vibration mill combined with ultrasonication. Deuterium-labeled CK internal standards (Olchemim) were added, each at 5 pmol per sample to check the recovery during purification and to validate the determination. The extracts were purified using a combined cation (SCX-cartridge) and anion (DEAE-Sephadex-C18-cartridge) exchanger and immunoaffinity chromatography based on wide-range specific monoclonal antibodies against cytokinins (Faiss et al., 1997). This resulted in three fractions: (1) the free bases, ribosides, and N-glucosides (fraction B), (2) a nucleotide fraction, and (3) an *O*-glucoside fraction. The metabolic eluates were evaporated to dryness and stored at −20°C until further analyses. CK fractions were quantified by ultra performance liquid chromatography (Acquity UPLC; Waters) coupled to a Quatro *micro* API triple quadrupole mass spectrometer (Waters) equipped with an electrospray interface. The purified samples were injected onto a C18 reversed-phase column (BEH C18; 1.7 μm; 2.1 ± 50 mm; Waters). The column was eluted with a linear gradient (0 min, 10% B; 0 to 8 min, 50% B; flow rate of 0.25 mL/min) of 15 mM ammonium formate, pH 4.0, (A) and methanol (B). Quantitation was obtained by multiple reaction monitoring of $[M+H]^+$ and the appropriate product ion. In MRM mode, the limit of detection for most of cytokinins was below 5.0 fmol, and the linear range was at least five orders of magnitude.

Tissue Regeneration

Tissue regeneration was done using a modified procedure based on previously described protocols (Ezura and Harberd, 1995; Catterou et al., 2002; Che et al., 2002). Plants were grown for 15 d on germination plates (half strength Murashige and Skoog [1962] supplemented with 1% saccharose, 0.5 g/L MES, and 0.8% agar). Root explants of 5 to 10 mm in length were then moved to CIM (Gamborg's B5 medium [Gamborg et al., 1968] with 0.5 g/L MES, 2% glucose, 0.2 μM kinetin, and 2.2 μM 2,4-D, 0.8% agar) and incubated for 5 d in continuous light. Finally, explants were either left on CIM or moved to root-inducing medium (Gamborg's B5 medium with 0.5 g/L MES, 2% glucose, and 0.9 μM 3-indoleacetic acid) or shoot-inducing medium (Gamborg's B5 medium with 0.5 g/L MES, 2% glucose, 0.9 μM 3-indoleacetic acid, and 0.5, 5, or 50 μM 2-isopentenyladenine) and incubated for 10 d in continuous light.

Accession Numbers

Arabidopsis Genome Initiative locus identifiers are as follows: *CDKA;1* (AT3G48750), *CDKB1;1* (AT3G54180), *CDKB2;1* (AT1G76540), *CDKB2;2* (AT1G20930), *CKS1* (AT2G27960), *CLV3* (AT2G27250), *CYCD4;1* (AT5G65420), *STM* (AT1G62360), *THIONIN 2.1* (AT1G72260), *TUBULIN BETA-2* (AT5G62690), and *WUS* (AT2G17950). The microarray data set has been deposited at ArrayExpress (EMBL-EBI) under accession number E-MEXP-1100.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Phylogeny and Expression Patterns of *Arabidopsis CDKs*.

Supplemental Figure 2. Expression of *CDKB2s* in Roots and during Callus Induction.

Supplemental Figure 3. In Situ Hybridizations with *CDKB2;1* and *CDKB2;2* 3′ Untranslated Region Probes.

Supplemental Figure 4. Alignment of *CDKB2* Sequences with Indication of amiRNA Target Sites.

Supplemental Figure 5. Phenotype of Hemizygous *35S:CDKB2;1* (OE1) Plants.

Supplemental Figure 6. Root Phenotypes of Wild-Type and *35S:CDKB2;1* (OE1) Plants.

Supplemental Figure 7. *CDKB2;1* and *CDKB2;2* Expression Levels in Transgenic Lines.

Supplemental Figure 8. Serial Sections through Apices of AM1-2 Plants Showing Multiple Expression Foci of Meristem Regulator Genes.

Supplemental Figure 9. Genotypes of F2 *WUS/35S:CDKB2;1* Plants.

Supplemental Table 1. Characterization of T1 *CDKB2* Transgenic Lines.

Supplemental Table 2. Primers Used to Amplify Open Reading Frames and amiRNAs.

Supplemental Table 3. Primers Used in q-RT-PCR Quantifications.

Supplemental Data Set 1. Genes Showing Significant Change in Expression Level between Wild-Type and Transgenic Lines.

ACKNOWLEDGMENTS

We thank Jürgen Berger for assistance with electron microscopy and Richard Clark, Kay Schneitz, Karin Schumacher, and Detlef Weigel for critically reading the manuscript. In addition, we thank Petra Amakorová for skillful technical assistance. This work was supported by the Max Planck Society, the SFB 446 funded by the Deutsch Forschungsgemeinschaft (J.U.L.); a Career Development Award of the Human Frontier Science Program and an EMBO Young Investigator Programme award to J.U.L.; a Carlsberg Foundation fellowship to S.U.A.; a fellowship of the Cusanuswerk to W.B.; and Grant MSM6198959216 of the Ministry of Education, Youth, and Sports of the Czech Republic to O.N.

Received July 30, 2007; revised December 18, 2007; accepted January 14, 2008; published January 25, 2008.

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